LIQUID MANURE AS A CULTURE MEDIUM FOR THREE SPECIES OF *CHLORELLA* (CHLOROPHYTA)

Jose Luis ARREDONDO-FIGUEROA[1], Graciela DE LARA-ISASSI[2] and Sergio ALVAREZ-HERNANDEZ[2]

Universidad Autónoma Metropolitana-Iztapalapa. Apartado Postal 55-535, México, D.F. 09340
[1] Planta Experimental de Producción Acuícola
[2] Laboratorio de Ficología Aplicada

ABSTRACT — Liquid sheep and cow manures were tested as alternative culture media for the growth of three species of *Chlorella* (Chlorophyta), and the efficiency of each was compared with that of the Bold-Basal medium. The efficiency of the three media was tested considering the cell density, the chlorophyll concentration and the nutritional value of the culture with respect to the content of lipids, carbohydrates and proteins. Results showed that the three algal species, *C. vulgaris*, *C. pyrenoidosa* and *C. minutissima*, grew in both liquid manures media as well as in the control medium. The analysis of the nutritional quality of each species showed no differences in the lipids, carbohydrates and proteins content. It can be concluded that liquid sheep and cow manures can be used as a low-cost alternative growth media for the production of these microalgal species for use in aquaculture.

RÉSUMÉ — Des excréments liquides de mouton et de vache ont été testés comme milieux de culture alternatifs pour la croissance de trois espèces de *Chlorella* (Chlorophyta). L'efficacité de chacun d'eux a été comparé avec celle du milieu de base de Bold. Cette efficacité a été évaluée à l'aide de la densité cellulaire, de la teneur en chlorophylle et de la valeur nutritionnelle de la culture au regard du contenu en lipides, hydrates de carbone et protéines. Les résultats ont montré que les trois espèces d'algues, *C. vulgaris*, *C. pyrenoidosa* et *C. minutissima*, se développaient dans les milieux à base d'excréments aussi bien que dans le milieu de contrôle. L'analyse de la qualité nutritionnelle de chaque espèce n'a montré aucune différence dans le contenu en lipides, hydrates de carbone et protéines. Il peut être conclu que les excréments liquides de mouton et de vache peuvent être utilisés comme milieu de culture alternatif pour la production de ces espèces de microalgues, en vu de leur utilisation en aquaculture. (Traduit par la Rédaction)

KEY WORDS: culture, *Chlorella*, freshwater microalgae, growth media, liquid manure.

INTRODUCTION

The development of fish, crustacean and mollusck cultures has created a growing interest in the mass production of some species of microalgae, for use as food at different stages of the life cycle of these animals (Ukeles, 1980). Microalgae constitute the

first link in the trophic chain that provide chemically varied nutrients (Laing & Millican, 1986; Whyte, 1987; Geldenhuys *et al.*, 1988) and, thus, either form part of the diet of some organisms or are used in the culture of rotifers, cladocerans and *Artemia* spp. which, in turn, serve as food in other stages of cultivation (Hirayama *et al.*, 1989).

Recently, there has been a surge of interest in the massive production of microalgae, and species with the best characteristics of growth, adaptability to the culture systems and satisfaction of the nutritional requirements of animal species, have been selected. Fábregas & Herrero (1985) demonstrated that four marine microalgae can be used as a potential source of single cell protein. They compared these algae with the amino acid composition of two freshwater microalgae and four animal protein sources and founded no significative differences among them.

At present, the quest for substances that act as fertilizers is ongoing. The optimum development of different species of microalgae is sought to lower the costs of culture media, mainly using agricultural chemical fertilizers or animal excrement that are provided either directly or in fermented form. These are known as liquid manure and contain important quantities of macronutients such as nitrogen (N), phosphorus (P) and potassium (K), as well as micronutrients such as calcium, copper, zinc, iron and magnesium (Taiganides, 1978). Liquid manure has a greater quantity of nitrogen than the original dry matter, with 85% of organic matter, 2.6% of nitrogen, 1.5% of phosphorus and 1% of potassium (Mandujano *et al.*, 1981). Thus, manure is an alternative of importance in the reduction of production costs; it does not alter the form and quality of the cells under culture; and production is as great as that registered with chemical fertilizers (Becker, 1986). Following the growing interest in aquaculture, which has focused mainly on the growth up of commercially important species in culture systems, it has become desirable to produce low-cost food of high nutritional value. This can be achieved using alternative growth media for production of natural food.

The purpose of this study was to test the efficiency of liquid manure as an alternative culture medium for the growth of three species of *Chlorella*, and to measure the growth and nutritional quality of each species with respect to protein, lipid, total carbohydrate and chlorophyll content.

MATERIALS AND METHODS

The efficiency of liquid cow and sheep manures as culture media and that of Bold-Basal as a control was tested using three species of Chlorophyta: *Chlorella vulgaris*, *Chlorella pyrenoidosa* and *Chlorella minutissima*. These were obtained from the collection of the Departamento de Biotecnología of the Universidad Autónoma Metropolitana Iztapalapa.

Cow and sheep excrement was biodigested for 20 days in 20 liters plastic buckets at room temperature. Once the liquid manure was formed, it was filtered to eliminate organic matter debris and big particles and sterilized in an autoclave at 15 lb for 15 minutes. In order to make the culture medium, 50 ml of liquid manure and 950 ml of previously sterilized distilled water were mixed. The Bold-Basal medium formulated by Nichols (1975) was used as a control to compare the efficiency of the liquid manures.

Three replicates of the cultures were prepared in previously sterilized 500 ml Erlenmeyer flasks, with a final volume of 250 ml (225 ml of medium and 25 ml of the

inoculum). The cultures were kept at 23°C and a 12:12 light:dark period, in a I-35 model Percival culture chamber. The cultures were both shaken using a New Brunswick Scientific Shaker at 150 r.p.m. and constantly aerated. The initial density of the cultures was 2.0 10^6 cells ml^{-1} for *Chlorella vulgaris* and *C. pyrenoidosa* and 2.1 10^6 cells ml^{-1} for *C. minutissima*. Cultivation was stopped on day 11 following recommendations of Richmond (1989), who suggested that in closed cultures, the entire biomass must be removed before the exponential growth phase is over. Growth curves were constructed with cell density data from samples taken daily for 10 days and counted using a Neubawer hematocytometer. Algae were harvested by centrifugation and then directly analyzed.

The nutritional quality of the cultured cells was established measuring proteins according to Lowry (1951), carbohydrates following the antrone method (Dubois *et al.*, 1956), and chlorophylls in accordance with Anonymous (1966). Total lipids were extracted following the method described by Bligh & Dyer (1959) and modified by Chiaverini (1972) which involves an extraction at low temperatures using a mixture of chloroform:methanol:water 2:1:1 v:v:v, followed by measurement according to the colorimetric method of Pandee *et al.* (1963).

The growth data of the microalgae in the three media were subjected to one-way parametrical analysis of variance (ANOVA), lineal regression and analysis of covariances (ANCOVA) using the STATISTICA release 4.5.

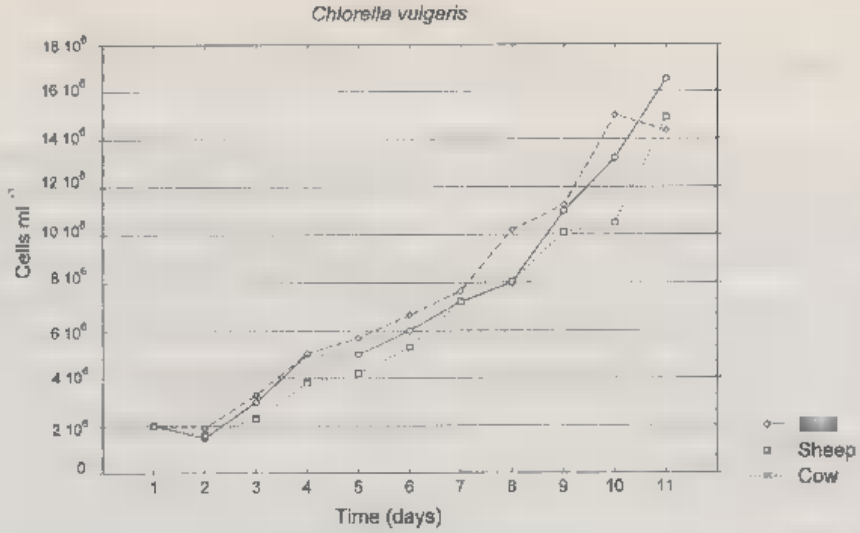
RESULTS

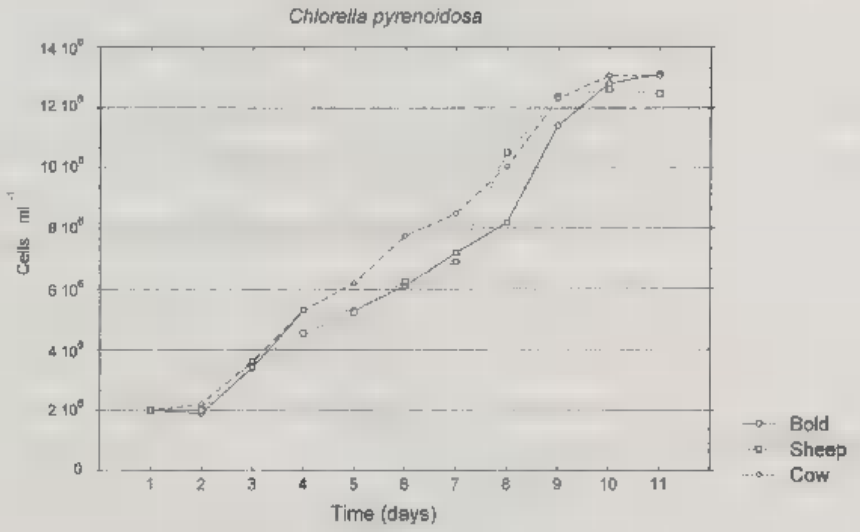
Growth in the Exponential Phase

The growth curves of *Chlorella vulgaris* in the three culture media showed maximum cellular densities at the end of the experiment (11 days) of 16.5 ± 0.16 10^6 cells ml^{-1} with the Bold-Basal medium, 14.9 ± 0.081 10^6 with liquid sheep manure and 14.35 ± 0.14 10^6 with liquid cow manure (Fig. 1). A decrease in cellular density was recorded for this last medium on the 11^{th} day of the experiment. Non-significant differences were recorded in the treatments by the ANOVA. In the case of *C. pyrenoidosa*, maximum densities were 13.1 ± 0.21 10^6 cells ml^{-1} with the Bold-Basal medium, 12.45 ± 0.18 10^6 with liquid sheep manure and 13.0 ± 0.047 10^6 with liquid cow manure (Fig. 2). The ANOVA results showed non-significant differences between treatments. Lastly, maximum densities of *C. minutissima* were 17.5 ± 0.074 10^6 cells ml^{-1} with the Bold-Basal medium, 18.5 ± 0.34 10^6 with liquid sheep manure and 19.25 ± 0.41 10^6 with liquid cow manure (Fig. 3). As in the other species, no significant differences were found in the ANOVA. The tests of slope of the regression curves (ANCOVA) in all treatments showed no significant differences.

Nutritional Content

The results of the bromatological analysis of the *Chlorella* species in the three culture media are presented in Table 1. Total chlorophyll values varied between 0.032 and 0.079 mg ml^{-1}, total protein content between 16.5 and 49.8 mg ml^{-1}, carbohydrates between 19 and 38 mg ml^{-1} and lipids between 7.9 and 20.8 mg ml^{-1}.





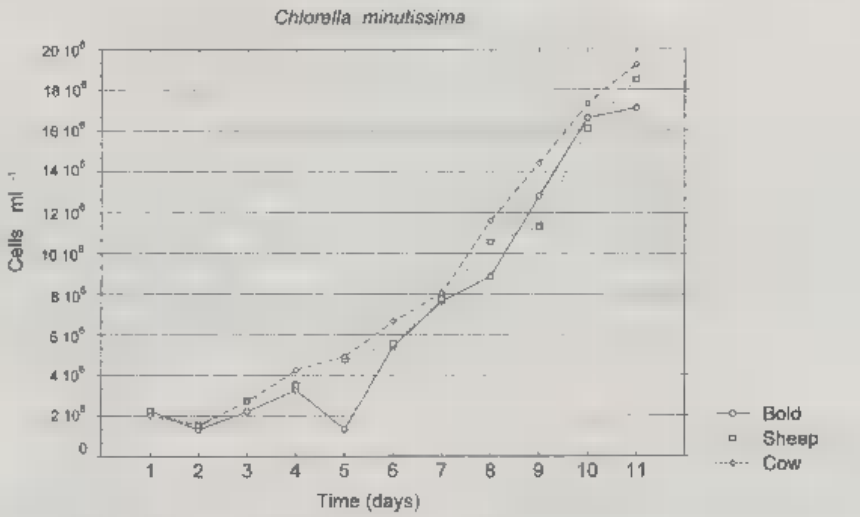


Fig. 1. Growth of *Chlorella vulgaris* with two alternative culture media (sheep and cow liquid manure) and a conventional medium (Bold-Basal), after an 11 days period.
Fig. 2. Growth of *Chlorella pyrenoidosa* with two alternative culture media (sheep and cow liquid manure) and a conventional medium (Bold-Basal), after an 11 days period.
Fig. 3. Growth of *Chlorella minutissima* with two alternative culture media (sheep and cow liquid manure) and a conventional medium (Bold-Basal), after an 11 days period.

Species	Culture medium	Chlorophyll *a* (mg ml^{-1})	Chlorophyll *b* (mg ml^{-1})	Total Chlorophyll (mg ml^{-1})	Proteins (mg ml^{-1})	Carbohydrates (mg ml^{-1})	Lipids (mg ml^{-1})
C. vulgaris	Bold-Basal	0.037 ± 0.002	0.04 ± 0.01	0.077 ± 0.001	49.8 ± 1	19 ± 0.1	11.5 ± 0.3
	Sheep liquid manure	0.024 ± 0.003	0.032 ± 0.01	0.056 ± 0.002	32 ± 0.9	28 ± 0.4	8.4 ± 0.4
	Cow liquid manure	0.022 ± 0.002	0.013 ± 0.003	0.035 ± 0.002	16.5 ± 0.6	19 ± 0.2	7.6 ± 0.4
C. pyrenoidosa	Bold-Basal	0.02 ± 0.006	0.029 ± 0.002	0.049 ± 0.001	49 ± 0.5	22 ± 0.5	10.2 ± 0.1
	Sheep liquid manure	0.02 ± 0.001	0.012 ± 0.006	0.032 ± 0.004	40 ± 0.3	20 ± 0.2	7.9 ± 0.1
	Cow liquid manure	0.033 ± 0.004	0.024 ± 0.002	0.057 ± 0.001	24 ± 0.4	30 ± 0.2	13.4 ± 0.1
C. minutissima	Bold-Basal	0.032 ± 0.003	0.047 ± 0.001	0.079 ± 0.003	38 ± 0.5	38 ± 0.5	9.6 ± 0.2
	Sheep liquid manure	0.031 ± 0.002	0.038 ± 0.003	0.069 ± 0.002	24 ± 0.8	29 ± 0.3	11.6 ± 0.2
	Cow liquid manure	0.04 ± 0.01	0.017 ± 0.002	0.057 ± 0.004	39 ± 0.4	33 ± 0.3	20.8 ± 0.1

Table 1. Bromatologic analysis of the *Chlorella* species with different growth media.

DISCUSSION

The use of animal excrement in culture media has provided good results with respect to cellular growth. Most research has been carried out with species that extract nutrients from processed pig excrement (Chung *et al.*, 1978; Soong, 1980; Noue & Basseres, 1989; Dabbadie, 1994), chicken, beef and human excrement (Shaalan *et al.*, 1989; Lincoln & Earle, 1990) with excellent results. The growth of the three species of *Chlorella* in both liquid cow and sheep manures was as good as that in the control medium. This is clearly showed in ANOVA and ANCOVA analysis where no significant differences were detected. *Chlorella minutissima* recorded the greatest cellular density in cow liquid manure, which coincides with data presented in the literature (Shaalan *et al.*, 1989; Lincoln & Earle, 1990). We propose the use of this culture medium as a low-cost alternative in the production of algal biomass. Lincoln & Earle (1990) found that only 50% of the nitrogen present in wastewater is recuperated by the algae and incorporated into proteins while almost 100% can be achieved with the standard medium, this being one of the main problems in the production of microalgae. Similar results were obtained by Shaalan *et al.* (1989) with *Chlorella pyrenoidosa*, the only difference being that their liquid cow manure was enriched in nitrogen, and proved that urea, added in low concentrations, is the best source of this macronutrient. Rathore & Kumar (1993) found that the protein content decreased during the last phases of the growth curve of *Chlorella vulgaris*, whereas carbohydrates and lipids remained constant during the experimental period. We recorded a maximum protein content in shorter culture periods (11 days). Our data for carbohydrates and lipids coincide with these authors results for this same species.

The size of the algae is important in the choice of food. Smaller algae induce a greater productivity of zooplanctonic organisms and filter-feeding fish, because the algae can be ingested easily (Lincoln & Earle, 1990). *Chlorella minutissima* is a species with a suitable diameter for consumption. The greatest cell density of this species was recorded with liquid cow manure; consequently we propose this culture medium as a good alternative in the mass production of the above-mentioned species.

REFERENCES

ANONYMOUS, 1966 — *Determination of photosynthetic pigments in sea water*. Monography no. 1. Paris, ONU, SCOR-UNESCO, 69 p.

BECKER W., 1986 — Nutritional properties of microalgae: Potentials and constrains. *In:* Richmond I. (ed.), *CRC Handbook of microalgal mass culture*. Boca Raton, Florida, USA, CRC Press, pp. 339-419.

BLIGH E.G. & DYER W.J., 1959 — A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology 37*: 911-917.

CHIAVERINI J., 1972 — *Techniques d'extraction et d'analyse des lipides, notes de travail N°. 12*. Université de Paris, Station Zoologique de Villefranche Sur-Mer, 12 p.

CHUNG P., POND W.G., KINGBURY J.M., WALKER E.F. & KROOK L., 1978 — Production and nutritive value of *Arthrospira platensis*, a spiral blue green alga grown in swine waste. *Journal of Animal Science* 47: 319-330.

DABBADIE L., 1994 — Results and knowledge obtained from intensive cultures of microalgae with pig manure. *Annales de Limnologie* 30: 233-245.

DUBOIS M., GILES K.A., HAMILTON J.K. REBERES P.A. & SMITH F., 1956 — Colorimetric method for determination of sugars and related substances, *Analytical Chemistry* 28: 350-356.

FABREGAS J. & HERRERO C., 1985 — Marine microalgae as a potential source of single cell protein (SCP). *Applied Microbiology and Biotechnology* 23: 110-113.

GELDENHUYS D.J., WALMSLEY R.D. & TOERIN D.F., 1988 — Quality of algal material produced on fertilizer-tap water medium outdoor plastic enclosed systems. *Aquaculture* 68: 157-164.

HIRAYAMA K., MARUYAMA Y & MAEDA.T., 1989 — Nutritional effect of freshwater *Chlorella* on growth of the rotifer *Brachionus plicatis*. *Hydrobiologia* 186/187: 39-42.

LAING I. & MILLICAN P.F., 1986 — Relative growth and growth efficiency of *Ostrea edulis* L. spat fed various alga diets. *Aquaculture* 54: 245-262.

LINCOLN E.P. & EARLE J.F.K., 1990 — Wastewater treatment with microalgae. *In*: Akatsuka I. (ed.), *Introduction to Applied Phycology*. The Hague, The Netherlands, SPB Academic Publishing, pp. 429-446.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. & RANDALL R.J., 1951 — Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 193: 265-275.

MANDUJANO A.M.I., FELIZ A.A. & MARTINEZ A.M., 1981 — *Biogas. Energia y fertilizantes a partir de desechos orgánicos*. Manual el promotor de la tecnología. Cuernavaca, México. OLADE: Serie Publicaciones Especiales no. 6, 41 p.

NICHOLS H.W., 1975 — Growth media-freshwater. *In:* STEIN, J.R. (ed.). *Handbook of Phycological Methods. Culture Methods and Growth Measurements*. New York, USA, Cambridge University Press, pp. 13-14.

NOUE J. & BASSERES A., 1989 — Biotreatment of anaerobically digested swine manure with microalgae. *Biological Wastes* 29: 17-31.

PANDEE S.V., KHAN R.P. & VANKITASUBRAMANIAN T.A., 1963 — Microdetermination of lipids and serum total fatty acid. *Analytical Biochemistry* 6: 415-423.

RATHORE D.S. & KUMAR. A., 1993 — Protein, lipid and carbohydrate content of three microalgae. *Phykos* 32: 9-12.

RICHMOND A., 1989 — Phototrophic Microalgae. *In:* Rehm H.-J. & Reed G. (eds). *Biotechnology. A Comprehensive Treatise Vol. 3*. Weinheim, Germany, Verlagsgesellchaft. pp. 109-143.

SHAALAN S.H., KHALEAFA A.F & KASEM A.M., 1989 — Algal single cell protein from extract of cow manure enriched with different nitrogen sources. *Bulletin of the National Intitute of Oceanography and Fisheries of Egypt* 15: 119-124.

SOONG P., 1980 — Production and development of *Chlorella* and *Spirulina* in Taiwan. *In:* G. Shelef & C.J. Soeder (eds), *Algal Biomass*. Amsterdam, Elsevier/North-Holland Biomedial Press, pp. 97-113.

TAIGANIDES E.P., 1978 — Principles and techniques of animal waste management and utilization. *FAO Soils Bulletin* 36: 341-362.

UKELES R., 1980 — American experience in the mass culture of microalgae for feeding larvae of the American oyster *Crassostrea virginica*. *In*: Shelef, G. & Soeder, C.J. (eds), *Algal biomass*. Amsterdam, Elsevier/North-Holland Biomedical Press.

WHYTE J.N.C., 1987 — Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. *Aquaculture* 60: 231-241.